REMARKS

Claims 1, 2, 5-7, 9, 12-14, 16-18, 20 and 47 are pending. The Examiner has withdrawn all previous rejections to the claims except the rejection under 35 U.S.C. §103(a). The Examiner is thanked for her careful reading of Applicants arguments and the withdrawal of previous rejections. Examiner has provided a detailed explanation of the basis of the remaining rejection under 35 U.S.C. §103(a). Applicants respectfully submit that the present rejection is based on a misunderstanding by the Examiner of the data in the cited art. To resolve some of these issues, Applicants thank Examiner Popa and Supervisor Woitach for the courtesy of a telephone interview on May 31, 2007. Applicants address the issues raised by the office action and in the interview below.

(1) The claimed method requires at least 20 fold higher ratio of RNaseIII to substrate (0.25:1) compared with Yang et al. to generate a plurality of overlapping fragments of a size of about 15-30 nucleotides.

Applicants' claimed method requires an enzyme substrate ratio of at least 0.25:1.

Yang et al. (US 2004/0014113) utilize enzyme:substrate ratios of about 0.001:1 where "100μg of dsRNA were digested by 1μg RNaseIII in a 200μl reaction buffer." (Yang et al. p. 8, ¶0079)

Yang et al. teach away from using increased amounts of RNAse III because "exhaustive cleavage of ds RNAse III leads to duplex products averaging 12-15bp in length" (Yang et al. p. 2 ¶0015).

Therefore Yang et al. teach a method of performing "limited RNase III digestion of dsRNA" (Yang et al.: p. 2, ¶0015). "Even at low RNaseIII concentrations, cleavage products were visible within 1 min incubation." (Yang et al. p. 6, ¶0053).

Gross et al. cleave substrate RNA under conditions described in Dunn et al. *J. Biol. Chem*. 251:3807-3814 (1976). However, it was not possible to deduce enzyme to substrate ratios from Gross et al. or from the cited Dunn et al. reference. Moreover, the dsRNA substrate used in Gross et al. is not comparable to the substrate used in Yang et al. because the substrate <u>before</u> cleavage is a very short dsRNA of 12bp maximum length (see Gross et al. Fig 3). In contrast the minimum size of the <u>cleavage product</u> of long ds RNA in Yang et al. is 12bp. [Applicants cleave large dsRNA substrate having a minimum size of 40bp (see Application, p. 25, line 6)].

The requirement in the claimed method of a ratio of at least 0.25:1 enzyme to substrate is supported by the description in the specification on p. 32 of the Application and also in Figures 1 and 2.

(2) RNaseIII cleaves dsRNA non-specifically

In the telephone conference on May 31, 2007 with the Examiner, there was some confusion about RNAseIII cleavage

specificity based on the teaching of Gross et al. which states that RNaseIII cleaves T7 ssRNA at 3 secondary sites in the presence of magnesium ions and at an additional 2 secondary sites in the presence of manganese ions where the T7 ss RNA is folded into a hairpin with "a short double stranded stretch of RNA" [no more than 12bp]. Gross et al. p. 432 ¶ I. Gross et al. did not suggest or teach cleaving large dsRNA into fragments of 15-30bp.

In contrast to the specific cleavage described by Gross et al., Yang et al. reported "the lack of sequence specificity in substrate recognition and cleavage by RNaseIII" (Yang et al. p. 6, ¶0053).

Applicants demonstrated non-specific cleavage of large dsRNA into 15-30bp fragments using RNaseIII in the presence of divalent transition metal cations (where "large" is defined as greater than about 40bp (Application pg 25, line 6)). In addition, Applicants showed by sequencing that the 15-30bp cleavage fragments were overlapping (Figure 4).

Yang et al. did not investigate whether 15-30bp fragments obtained by RNaseIII cleavage of a dsRNA template in the presence of magnesium ions were overlapping. Nonetheless, the size fractionation of the cleavage products by Yang et al. to remove large dsRNA cleavage fragments (Yang et al., Figures 1B and 1C) likely affects the distribution of cleaved fragments of the target size with respect to the template dsRNA. There is no suggestion in the art as to how short ss RNA that form hairpin substrates described by Gross et al. interact with RNAseIII and manganese and how this compares with cleavage

of large dsRNA in the presence of divalent transition metal cations. Certainly, any suggestion of specific cleavage sites in Gross et al. teaches away from the cleavage pattern demonstrated by Applicants in Figure 4 of the Application.

(3) Neither Yang et al. nor Gross et al. separately or together suggest that the problem of exhaustive cleavage of large dsRNA into fragments of 12-15nt could be solved by replacing magnesium ions with a divalent transition metal cation.

As discussed above, Yang et al. teach that exhaustive cleavage noted in the p. 2, ¶0015) can be resolved by "limited" RNaseIII digestion. "Limited RNaseIII digestion" was achieved by Yang et al. using short incubation times and low concentrations of RNaseIII relative to substrate.

Gross et al. did not address the problem of exhaustive digestion of large ds RNA. The short ds RNA regions in the hairpins were less than 12 bp (see Figure 3 of the reference). Hence, the problem that was addressed was different, namely, how to introduce, instead of reduce, cleavage sites in very short regions of short dsRNA. Gross et al. state that:

> *E. coli* RNaseIII introduces homogeneous cuts [into viral RNAs in vitro].... at a relatively short stretch of base pairing in ssRNA. Additional secondary sites are cut in the presence of manganese...instead of magnesium..ions. [emphasis added] (Gross et al. p. 432, ¶ I).

Consequently, Gross et al. teaches away from Yang et al.

(4) The claimed composition requires that the *in vitro* cleavage product of large dsRNA represents a substantial portion of dsRNA sequence and that the fragments comprise a plurality of overlapping fragments of about 15-30 nucleotides

Applicants developed a method of cloning fragments obtained from dsRNAseIII digestion in the presence of a divalent transition metal cation that enabled the fragments to be sequenced and mapped to the sequence of the large dsRNA substrate (Figure 4). In addition, Applicants conducted radio-labelled RNA hybridization studies to demonstrate that a significant portion of the isotope mapped to fragments in the size range of 15-30nt (Figures 3A and 3B). No such studies were performed by Gross et al. or Yang et al. and hence it could not be determined whether RNAseIII reaction products are overlapping.

Rebuttal of Examiner's specific comments

The Examiner suggests that Yang et al. have identified the conditions necessary for digesting large dsRNA into cleavage fragments of size 15-30 bps. However, Yang et al. achieved this result by limited digestion of substrate in short periods of time and then size fractionating the product to obtain a preparation of dsRNA of defined size.

In contrast, Applicants claim an improved method of preparing dsRNA of a defined size (15-30bp) by incubating RNAseIII in a reaction buffer containing a divalent transition metal cation such as manganese using a ratio of enzyme to substrate of 0.25:1. The

transition metals have incomplete D-orbitals. Magnesium which is an alkaline earth metal, is generally more reactive than transition metals. Applicants surprisingly discovered that the use of divalent transition metal ions in place of magnesium resulted in an enriched population of cleavage fragments of 15-30bp that did not require size fractionation required by Yang et al. to remove toxic larger fragments. The cleavage fragments additionally were shown by Applicants to overlap in sequence and constitute a substantial portion of the large dsRNA.

(a) The Examiner states on p. 6 of the office action dated January 4, 2007 that:

> Yang et al. do teach that 15-30 bp are visible within 1 minute and that by 3 minutes these fragments *become the main products* (i.e. complete digestion) when the incubation is performed at 37°C (p. 6, ¶ 0053, Figure 1b, line 6).

The Examiner correctly notes an incubation of 1 and 3 minutes in Yang et al. However, Figure 1b of the reference shows a smear that includes smaller and large fragments within the "main products".

(b) The Examiner states on p. 6 of the office action dated January 4, 2007 that

> Fig. 1B represents an agarose.... ... lanes 1, 2, 4, 5, 7 and 8 (i.e. the gradients of bands with different sizes or smears) are dsRNA to be digested (it is known in the art that RNA migrates as a smear on agarose gels) while lanes 3, 6 and 9 are esiRNA. It is clear from the figure that esiRNA migrates as a well-defined band and *therefore a limited digestion does not result in a smear.*

Applicants respectfully submit that the statement by the Examiner is not consistent with the description of Figure 1 where Figure 1B describes the product of initial digestion with RNaseIII in the presence of magnesium and Figure 1C shows size fractionation to produce esiRNA fragments as defined on p. 6, ¶0053.

On p. 1, ¶ 0005, Yang et al. state:

FIGS. 1A-C:

> (b) Time course of RNase III digestion of dsRNA. DsR-457 or dsF-592 RNA was incubated with RNase III for indicated time, and then separated by electrophoresis in a 4% agarose gel.
>
> Lanes 1, 4 and 7 are dsR-457;
> Lanes 2, 5 and 8 are dsF-592;
> Lanes 3, 6 and 9 are 21 bp **siRNA*** marked by an arrow.
>
> (c) Agarose gel analysis of **purified short RNA species processed from dsF-592**. Lane M, 10 bp DNA marker; Lane 1 and 2 are chemically synthesized siRNAs. Lane 3, 21-23 bp; lane 4, 24-26 bp; Lane 5, 27-30 bp.

Please note that ¶0005 states that lanes 3, 6 and 9 are siRNA not esiRNA hence it should be assumed that these size markers were made by chemical synthesis because ¶0053 states that only siRNA used for gene silencing is called esiRNA..

The "processing" described in p. I, ¶0005 is further elaborated in p. 6, ¶0053 of the Yang et al. reference, which describes

"Processing long dsRNAs into short RNA duplexes using E. coli RNaseIII" as including a size separation step.

> (¶0053)We separated RNaseIII-digestion products on polyacrylamid gels and **purified** the RNAs corresponding to approximately 21-23, 24-26, and 27-30 bp (FIG. 1c). For simplicity, we named siRNA prepared by RNase III digestion as esiRNA (endoribonuclease-prepared siRNA).

(c) The Examiner states on p. 5 of the office action that: " [a]pplicant's assumption that RNAi appears to work only sometimes is incorrect."

Applicants comments on p. 17 of the response dated October 4, 2006 refer to the Yang et al reference, where it states: "ample amounts of esiRNA for inhibition of most [but not all] genes" (Yang et al. p. 6, ¶0053).

(d) The Examiner has addressed the issue of efficiency of cleavage by RNAseIII as "more efficient cleavage" and not "generation of smaller unsuitable fragments" citing Fig 1C of Yang et al. However, on page 2, 0014, Yang et al states: "E.coli RNAseIII can digest dsRNA very efficiently into short pieces" and p. 2, ¶ 0015 Yang et al states " Exhaustive cleavage of dsRNA by E.coli RNaseIII leads to duplex products of 12-15bp in length" Hence Yang et al. teach "limited digestion."

> Gross et al. teach more efficient cleavage and not generation of smaller unsuitable fragments.....one of ordinary skill in the art would have known that using the digestion conditions taught by Gross et al. would not result in unsuitable fragments for RNAi. Moreover one of ordinary skill in the art would have

> been motivated to change the method of Yang et al. by using the digestion conditions of Gross et al. .....would have expected a more efficient digestion. (p. 7 of the office action dated 1/4/07)

However, cleavage of short dsRNA hairpins (less than 12bp) embedded in ssRNA described by Gross et al is different from the dsRNA cleavage described in Yang et al.. Yang et al. cleaves "long" dsRNA fragments into smaller fragments (Yang reference p. 2, ¶ 0014)

(e) The Examiner states on p. 8 of the office action that:

> The art teaches the utility of using a range of different ratios for identification of optimal reaction conditions and that this can be accomplished by routine experimentation.

The routine experimentation suggested by Yang et al. is to use low ratios of enzyme to substrate to avoid excess cleavage of the substrate. This teaches away from a relatively high ratio of enzyme:substrate required in the claimed method. The Gross reference does not provide enzyme substrate ratios and is silent on the effect of varying enzyme concentration.

SUMMARY

I. The claimed method and composition are not suggested or taught by Yang et al. in view of Gross et al. nor are they predictable from the combination of the two references

The teachings of Yang et al. are not compatible with Gross et al. Yang et al. seek to control the time of digestion of substrate using low concentrations of RNaseIII and magnesium ions to prevent the exclusive formation of fragments of size no greater than 12-15bp in length so as to collect by fractionation a transient population of dsRNA fragments of 15-30 bp suitable for gene silencing.

Gross et al. do not speak to the concentration of RNaseIII. Instead Gross et al. describe how to cleave additional sites in very short sequences of dsRNA (no greater than 12 nucleotides) contained in hairpin folded single stranded viral RNA.

Taken together, these references do not provide a basis for predicting how to produce overlapping dsRNA fragments of size 15-30 bp using RNaseIII in the presence of divalent transition metal cations that preferentially rely on a ratio of enzyme to substrate of at least about 0.25:1.

II. It was not possible to anticipate a reasonable likelihood of success in making siRNA molecules from Yang et al. in view of Gross et al. even in the unlikely situation where these references were sought to be combined despite their described different purposes and goals. This is because the references teach away from each other.

The use of relatively high concentrations of RNAseIII (0.25:1) in the claimed method is counterintuitive with respect to combination of Gross et al with Yang et al. This combination of references would suggest low concentrations of RNAseIII for preparing dsRNA to reduce cleavage (Yang et al) and low concentrations of manganese ions to increase cleavage of small dsRNA at additional sites.

III. Claim 13 and dependent claims could not be predicted from combining Yang et al. with Gross et al.

Neither Yang et al. nor Gross et al. suggest a purified set of double stranded RNA fragments that are overlapping and represent a substantial portion of the sequence of the template RNA. Indeed it would appear unlikely that the method of Yang et al which cleaves the dsRNA into a spectrum of sizes over a short time period and subsequently into fragments of 12-15bp such that size fractionation is utilized to obtain fragments of the desired size

CONCLUSION

For the reasons set forth above, Applicants respectfully submit that this case is in condition for immediate allowance. Early and favorable consideration leading to prompt issuance of this Application is earnestly solicited.

Applicants petition for a three-month extension of time to file a response and enclose a notice of appeal. A check in the amount of $760 is enclosed, covering the fees for the extension and notice. Please charge any deficiencies or credit any overpayment to Deposit Account No. 14-0740.

Respectfully submitted,

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